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Abstract

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Keywords

Influenza A virus, Neuraminidase inhibiting antibodies, Live attenuated influenza virus, Whole inactivated virus, Mucosal immunity, Maternally derived antibodies

Disciplines

Large or Food Animal and Equine Medicine | Veterinary Infectious Diseases | Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Comments

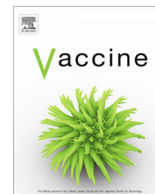
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ABSTRACT

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1. Introduction

The neuraminidase (NA) protein of influenza A virus (IAV) has sialidase activity that facilitates the release of new viral particles from receptors on infected cells [1,2]. NA inhibitor drugs that bind to the sialidase catalytic site are widely used therapeutics for influenza patients [3]. Likewise, antibodies targeting NA have been correlated with reduced disease severity and viral replication in animal models [4,5]. Human clinical studies have also shown evidence for reduced disease severity in individuals with well-matched circulating NI antibodies [6–8]. NA antibody-binding sites

undergo antigenic drift comparable to hemagglutinin (HA) antigenic drift [9,10], suggesting that NA-specific antibodies exert selective pressure for mutations. Mechanisms for the protective action of NA-specific antibodies were investigated soon after NA enzymatic function was reported. Strain-matched NA antiserum added to *in vitro* IAV culture reduced the virus yield from infected cultures and reduced the size of plaques [11]. Electron microscopy showed that NA-specific antiserum promotes aggregation of new IAV viral particles on the surface of infected cells [2,12], hindering the spread of virus to other cells.

Research characterizing NI antibody responses to vaccination has been fairly limited in any host, especially concerning live-attenuated influenza virus (LAIV) vaccines. Published studies of NI responses in pigs are extremely limited. We hypothesized that intranasal LAIV vaccines, like natural infection, elicits both local and systemic NI antibodies. NI antibodies at the respiratory mucosal surfaces might be particularly effective at reducing viral replication *in vivo*. A recent study analyzed NI activity in nasal

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secretions of human subjects before and after an influenza season [8]. Nasal NI titers were statistically correlated with reduced severity of natural infection during the subsequent months. Another recent clinical study identified an inverse correlation between serum NI antibody responses induced by seasonal IAV vaccines and the incidence of seasonal influenza infection [13]. In human clinical studies it is usually not possible to discern whether pre-existing NI antibodies were induced by infection or vaccination or the combination. In human subjects it is also difficult to collect specimens suitable to analyze influenza antibodies in the lungs. Animal studies with natural IAV host species, such as pigs, offer the possibility to measure local NI antibodies of the upper versus lower respiratory tract.

Swine IAV is an economically important pathogen affecting pig populations around the globe [14]. Swine IAV also has public health significance, exemplified by the emergence of swine-origin 2009 pandemic H1N1 virus and zoonotic H3N2 virus infections at agricultural fairs [15–17]. Furthermore, since the pig is a natural IAV host, it is a valuable experimental model for testing vaccines and immune parameters relevant to human IAV. There are multiple IAV lineages co-circulating in pigs [14], belonging to subtypes H1N1, H1N2, and H3N2. Hemagglutinins (HA) of the swine H1 and H3 subtypes are genetically and antigenically diverse [18,19]. There is also diversity in NA genes. Contemporary North American swine N2 genes fall into two distinct lineages, one acquired by reassortment with a human seasonal H3N2 strain detected in pigs in 1998, and the other acquired by reassortment with a more contemporary human seasonal H3N2 from about 2002 [20]. Thus, we also hypothesized that these distinct genetic differences in N2 genes would alter antibody cross-reactivity directed against the NA proteins.

Current commercial vaccines against swine IAV (containing whole inactivated virus (WIV) plus adjuvant) do not give reliable protection against the diverse co-circulating viruses [21]. Intranasal LAIV vaccines attenuated by several mutation strategies have shown efficacy against experimental challenge with heterologous strains [22–24], and in maternal antibody-positive piglets [25]. LAIV vaccines in swine have been shown to induce serum neutralizing antibodies, virus-binding IgA in the respiratory tract, and cell-mediated immunity [26]. In the current study we quantified vaccine-induced NI antibodies in serum and respiratory mucosal specimens, examined the antigenic cross-reactivity between the two phylogenetic lineages of N2 subtype NA, and analyzed NA-specific maternal antibodies in piglets receiving colostrum from vaccinated sows.

2. Materials and methods

2.1. Viruses

Virus strains used to generate vaccines were a delta-1 cluster H1N2 isolate, A/swine/Minnesota/02011/2008 (MN08), and a

cluster 1 H3N2 isolate, A/swine/Texas/4199-2/1998 (TX98) (Table 1). In experiment 1, pigs received heterologous challenge infection with the 2009 pandemic strain A/California/04/2009 (H1N1). Test antigens for use in the NI assay consisted of viruses derived by reverse genetics, by previously reported methods [27], and cultivated in MDCK cells. The HA genes included in the reverse-engineered NI antigens were intentionally mismatched to the HA in the vaccine viruses to rule out antibodies binding to HA at the virus surface and interfering non-specifically with NA function. One virus (H9N2_{MN08}) was a reassortant containing the six internal gene segments of A/Puerto Rico/8/1934, the HA gene of A/guinea fowl/Hong Kong/WF10/1999 (HK99)(H9N2), and the NA gene of MN08, representing the 1998 N2 lineage. A second reassortant virus (H9N2_{OH04}) had the HA gene of HK99 and all other gene segments of OH04, including NA of the 2002 N2 lineage. A third reassortant virus (H1N2_{TX98}) contained the six internal gene segments and HA of A/Puerto Rico/8/1934 and the NA gene of TX98.

2.2. Experimental design

2.2.1. Vaccination experiment 1

MN08 vaccines were administered as previously described [28]. Briefly, an LAIV vaccine was generated using reverse genetics to insert the MN08 H1 and N2 gene segments into the attenuated A/turkey/Ohio/313053/04 (OH04) backbone [24]. Immunization by wild type (WT) virus infection was done using live MN08. The whole inactivated virus (WIV) vaccine was UV-irradiated MN08 mixed with a commercial oil-in-water adjuvant (Emulsigen D; MVP Laboratories, Inc., Ralston, NE). The sham vaccine (SV) was prepared from cell culture supernatant mixed with Emulsigen D. Pigs were cared for in accordance with the Institutional Animal Care and Use Committee (IACUC) of the National Animal Disease Center. Experimental groups received two doses of their respective vaccine at 4 and 7 weeks of age. Those in the LAIV and WT groups were vaccinated intranasally with 2 ml of virus at 10^6 50% tissue culture infectious doses (TCID₅₀) per ml. The WIV vaccinated pigs were administered 2 ml (128 HA units) of inactivated, adjuvanted vaccine by intramuscular injection. Negative controls received 2 ml of SV by the same route. The sera tested by enzyme-linked lectin assay (ELLA) were collected 6 weeks post-primary vaccination and heat-inactivated at 56 °C for 30 min. Pre-challenge bronchoalveolar lavage (BAL) fluid and nasal wash (NW) samples were collected from subgroups of pigs ($n=8$) that were humanely euthanized 6 weeks post-primary vaccination. Remaining pigs in each group ($n=8$) were challenged 6 weeks post-primary vaccination by intranasal infection with A/California/04/2009 at 1×10^3 TCID₅₀/ml. These pigs were euthanized and BAL fluid was collected at 5 days post-infection (dpi) to compare pre- and post-challenge NI responses to heterologous challenge. Fifty ml MEM was pipetted into lungs via the trachea, followed by a series of 3–4 repeated lavages, and approximately 15–20 ml recovered in

Table 1
Summary of the components of vaccine, challenge and neuraminidase inhibition test viruses.

Animal study	Vaccines		Challenge virus	HA/NA of NI test antigens
	Platform	HA/NA strain		
1	LAIV	MN08 ^a H1N2 (1998-lineage N2)	CA09 ^b (H1N1)	H9N2 _{MN08} (1998-lineage N2)
	WIV			H1N2 _{TX98} (1998-lineage N2)
	WT			H9N2 _{OH04} (2002-lineage N2)
2	LAIV	TX98 ^c H3N2 (1998-lineage N2)	N/A ^d	H1N2 _{TX98} (1998-lineage N2)
	WIV			

^a A/swine/Minnesota/02011/2008.

^b A/California/04/2009 (pandemic).

^c A/swine/Texas/4199-2/1998.

^d Challenge virus not addressed in this study.

the final specimen. BAL fluid specimens from infected pigs were incubated with turkey erythrocytes on ice to absorb virus that might affect the ELLA readout.

2.2.2. Vaccination experiment 2

As described previously [26], piglets with or without vaccine-matched passive maternal antibodies were immunized with WIV or LAIV vaccines. Briefly, sows were vaccinated intramuscularly with Emulsigen D-adjuvanted TX98 WIV in 3 doses, before and during pregnancy. Co-housed control sows received no IAV vaccine. After farrowing, one week-old piglets were bled to collect serum and confirm transfer of IAV-specific maternal antibodies. At 14–19 days of age piglets were weaned and randomly assigned to vaccine treatment groups within the appropriate maternally derived antibody (MDA) status, and initial doses of WIV as given to the sows or LAIV were given at 15–20 days of age. The TX98 LAIV was generated via reverse genetics as previously described [29], with the NS1 gene truncated to encode 126 amino acids. Piglets were immunized with 2 ml of LAIV at 10^6 TCID₅₀/ml intranasally or 2 ml WIV intramuscularly. Booster doses were administered 2 weeks later to the WIV and LAIV groups.

2.3. Enzyme-linked lectin assay (ELLA) for neuraminidase antibodies

Serum and BAL fluid neuraminidase inhibition (NI) titers were measured using an assay described previously [30,31]. Briefly, NA enzymatic activity of a virus was quantified by sialic acid cleavage from fetuin on 96-well plates during an overnight incubation at 37 °C. Peanut agglutinin-horseradish peroxidase conjugate (PNA-HRP) was then added for 2 h at room temperature, binding to fetuin molecules stripped of sialic acid. Signal was obtained with O-phenylenediamine dihydrochloride (OPD) substrate, and read at 490 nm. Test antigens were titrated to determine the highest dilution able to yield maximum signal. For NI antibody titration, serial dilutions of serum or BAL fluid were mixed with virus at equal volumes in duplicate fetuin-coated wells during the overnight 37 °C incubation. Optical density (OD) values were normalized to the values from positive control wells containing no serum. Non-linear regression, with log₂ transformed serum dilutions as the independent variable, was performed to calculate the serum dilution that would inhibit 50% of NA activity (IC₅₀) (GraphPad Prism software). Spearman correlation coefficients were calculated for homologous HI and NI titers within each treatment group from vaccination experiment 1, using GraphPad Prism software. Statistical significance of correlation was determined with a two-tailed *p*-value ($\alpha = 0.05$).

3. Results

3.1. Neuraminidase antibodies induced by vaccination

In the first vaccination experiment we administered WIV and LAIV vaccines and the WT virus to naïve piglets (Table 1). Viruses in each vaccine formulation carried the NA gene of A/swine/Minnesota/02011/2008 (MN08) (H1N2). NA_{MN08} belongs to the 1998 N2 lineage (Fig. 1). Groups that were immunized with WIV, LAIV, or WT virus had robust serum NI responses to the homologous NA antigen (Fig. 2A). The background titers in sham-vaccinated animals ranged between 5 and 40. In contrast, all three immunized groups developed geometric mean homologous NI titers between 640 and 1280. The highest and most consistent response was in piglets infected with live WT MN08, but there were no statistically significant differences among the immunized groups. All of the vaccines also induced robust homologous HI

antibody responses, and the homologous NI titers were plotted against homologous HI titers (Fig. 3). In the LAIV and WT groups there were statistically significant correlations between NI and HI titers, with *r* values of 0.609 (*p* = 0.0140) and 0.523 (*p* = 0.0385), respectively. However, the WIV vaccinated group displayed more variability in the NI response compared to the HI response, resulting in a non-significant *r* value of 0.425 (*p* = 0.102). The WIV vaccine dose was standardized to units of HA activity, and since the inactivated viruses do not replicate to increase the NA protein quantity, the response to the WIV was likely biased toward the HA.

NI titers were also determined against two heterologous NA antigens. NA of the H3N2 strain A/swine/Texas/4199-2/1998 (TX98) also belongs to the 1998 N2 lineage, but it preceded the N2 of the MN08 by ten years and shared 96.8% amino acid sequence identity with the NA_{MN08} present in the experimental vaccines (Fig. 1). Cross-reactive serum antibodies to NA_{TX98} were readily detectable in all MN08-immunized groups (Fig. 2B). The geometric mean titer in those three groups was slightly below 160, with heterologous NI titers generally 4- to 8-fold lower than homologous titers to NA_{MN08}. The other heterologous antigen contained NA of the H3N2 strain A/turkey/Ohio/313053/04 (OH04), which falls into the 2002 N2 lineage (Fig. 1). The NA amino acid sequence identity between OH04 and the vaccine strain, MN08, is 91.9%. Cross-reactive serum NI titers against NA_{OH04} were mostly undetectable (Fig. 2C).

BAL fluid and nasal wash specimens were collected 6 weeks post-primary vaccination from the same pigs as the sera. In pigs vaccinated with intramuscular WIV or sham vaccine, BAL fluid NI titers were not detectable (Fig. 4A). In contrast, groups that had been exposed to live LAIV or WT MN08 virus via the respiratory route developed homologous NI titers in lungs. The results were very similar between the LAIV and WT groups, with geometric mean titers of 23.8 and 27.2, respectively. In these groups there was considerable variation, with titers ranging from near the lower limit of detection (5) to above 100. Cross-reactive NI titers to the heterologous NA_{OH04} antigen, representing the 2002 N2 lineage, were essentially undetectable in BAL fluid specimens (data not shown). Other pigs in each of the four treatment groups underwent heterologous challenge with CA09 (pandemic H1N1), with outcomes reported previously [28]. With respect to NA proteins this was a heterosubtypic challenge virus, but we asked if IAV infection of vaccinated pigs would increase MN08-specific NI antibodies in the lungs. Titration of 5 dpi BAL fluid showed that geometric mean NI titers in lungs of the LAIV and WT groups reached considerably higher levels than before challenge: 256 and 429, respectively (Fig. 4B). Modest NI titers also appeared in BAL fluid of WIV-vaccinated, CA09-infected pigs, but the geometric mean titer remained below 20.

Nasal washing is more practical for routine sampling of the respiratory mucosa, as it is performed without euthanizing or anesthetizing pigs, so we tested whether NI titers were present in nasal secretions (Fig. 4C). Nasal wash specimens from immunized pigs had relatively weak NI reactivity against the homologous NA_{MN08} antigen. There was a statistically insignificant trend for higher nasal NI activity in the LAIV and WT virus treatment groups, where most of the animals had titers of at least 10. In contrast, a titer of 10 was recorded for only one WIV-vaccinated pig and none of the SV controls. Only the WT MN08 group had nasal NI activity statistically different from the SV control group, albeit a modest difference, with geometric mean titers of 12.0 and 7.9, respectively. Background NI activity of nasal wash samples was higher than in BAL fluid. In all, there was evidence that NI antibodies can be detected in the upper respiratory tract after pigs are exposed to a live virus, but titers there are lower in magnitude and more difficult to distinguish from background inhibition.

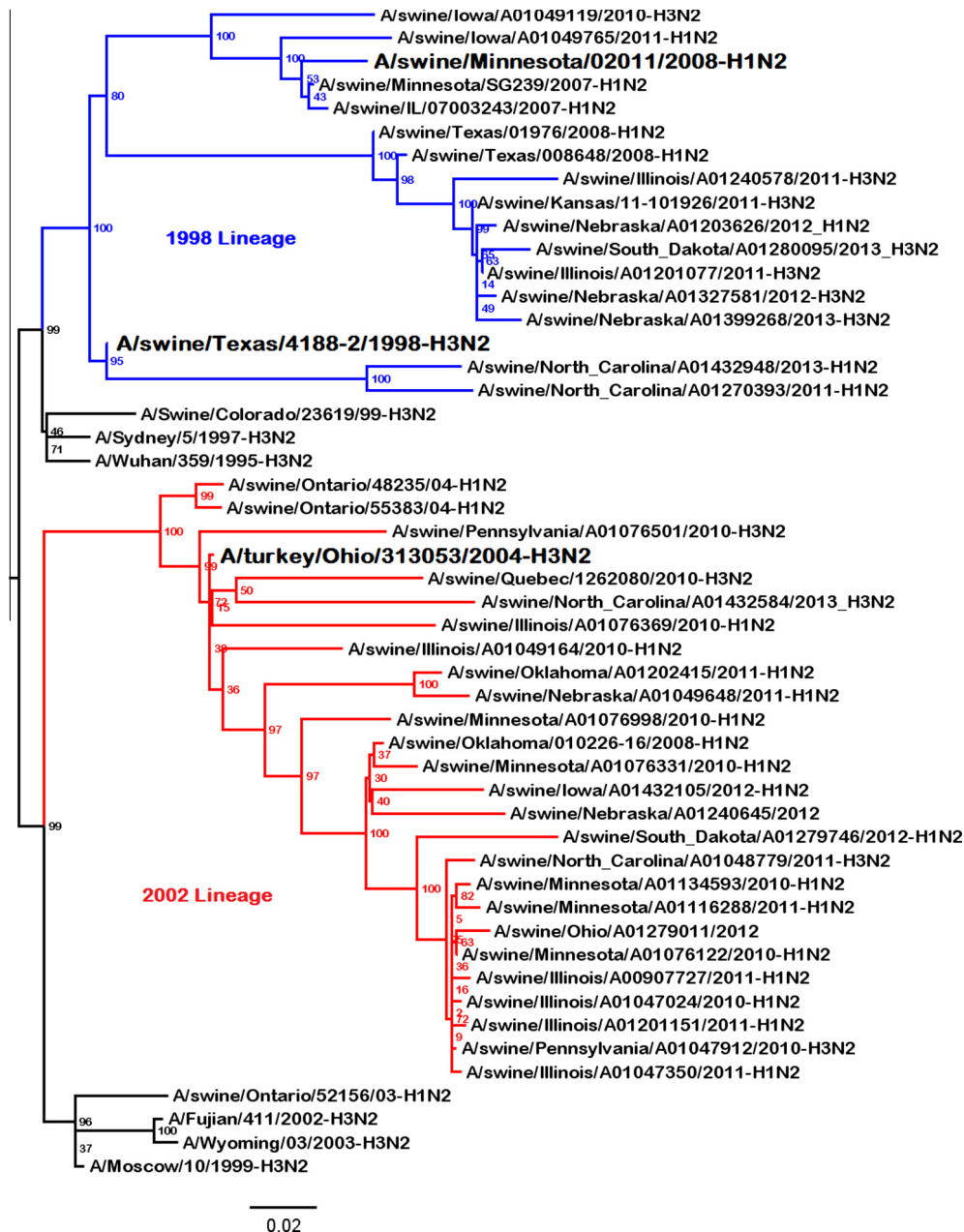


Fig. 1. N2 genes representing two neuraminidase lineages in North American swine since 1998. Included in the maximum likelihood tree are N2 genes of five human strains selected for contemporaneous seasonal influenza vaccines. The three swine strains supplying N2 antigens for NI assays in this study are printed in bold.

3.2. Passive maternally-derived neuraminidase antibodies

A second vaccine study quantified NA-specific maternal antibodies in piglets and also tested the impact of passive, maternal immunity on active NI antibody responses to vaccination. Sows were immunized before and during gestation with WIV vaccines containing the TX98 strain. Piglets from WIV vaccinated sows obtained abundant amounts of circulating NI antibody (Fig. 5A). On the day of weaning and LAIV vaccination, when piglets were 15–20 days of age, the geometric mean serum NI titer was 2417. The background serum inhibition of piglets from naïve sows was very low. Likewise, serum hemagglutination inhibition (HI) titers were abundant in piglets of the vaccinated sows and undetectable in piglets from naïve sows [26]. The NI assay was repeated with serum specimens collected 42 days later, after piglets had received 1 or 2 doses of TX98 LAIV vaccine. In the non-vaccinated (NV)

group the high passive NI antibody titers had waned markedly during those 6 weeks to mostly undetectable levels (Fig. 5B); this was comparable to the waning of HI titers reported previously [26]. Maternal antibodies partially blocked serum NI responses to intranasal LAIV vaccination (Fig. 5B). Even in maternal antibody-negative groups, the serum NI responses to 1 or 2 doses of LAIV vaccine were modest (geometric mean titers of 36.6 and 32.8, respectively), but they were significantly higher than titers of their maternal antibody-positive counterparts and the non-vaccinated groups.

4. Discussion

Inactivated IAV vaccines licensed for humans and for pigs induce circulating antibodies that neutralize antigenically identical

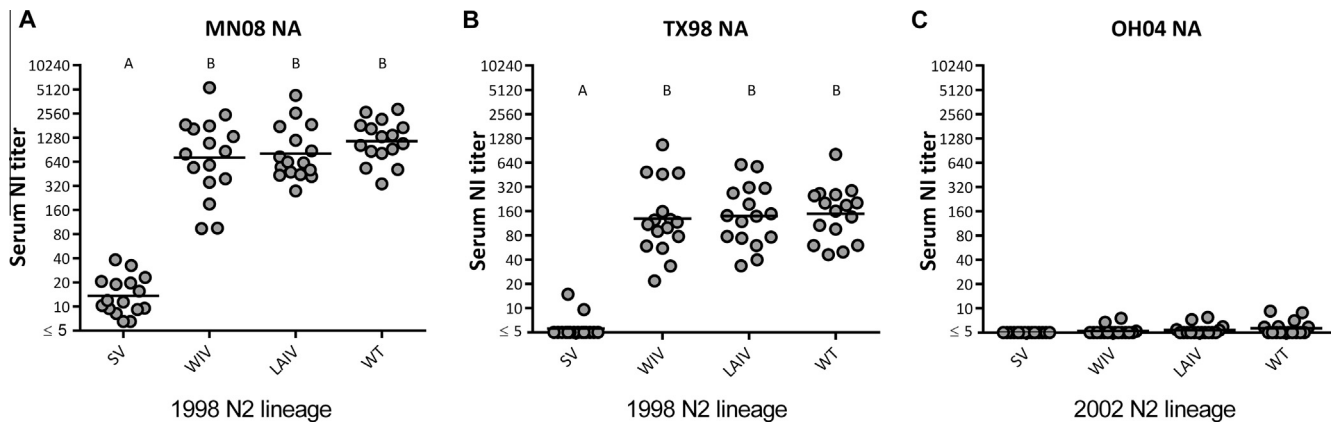


Fig. 2. Functional serum NI antibodies in pigs following vaccination or infection. Three weeks after the administration of secondary doses (six weeks post-primary vaccination) sera were collected. NI antibody titers to the homologous 1998-lineage N2_{MN08} (A), heterologous 1998-lineage N2_{TX98} (B), and heterologous 2002-lineage N2_{OH04} (C) proteins were determined by the ELLA method, using reverse genetics-derived reassortant viruses as test antigens. Titers represent 50% enzymatic inhibition endpoints as calculated by nonlinear regression. Data were analyzed statistically by ANOVA and Tukey's pairwise comparison test. Treatment groups marked with different letters had significantly different titers ($p < 0.05$).

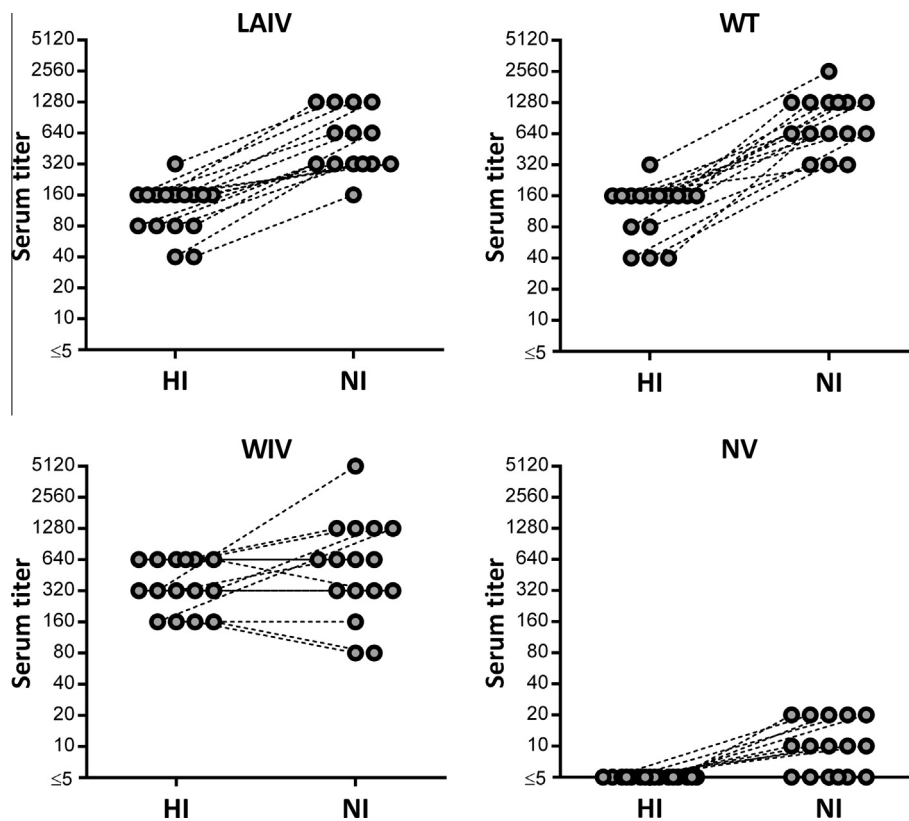


Fig. 3. Comparison of homologous neuraminidase inhibition titers to homologous hemagglutination inhibition titers. For each vaccine treatment group in experiment 1, serum NI titers to homologous 1998-lineage N2_{MN08} antigen were plotted against the corresponding homologous HI titers. In the LAIV, WT, and WIV vaccinated groups there were Spearman correlation coefficients of 0.609 ($p = 0.0140$), 0.523 ($p = 0.0385$), and 0.425 ($p = 0.102$), respectively.

or similar strains, while offering limited protection against antigenic drift variants and newly emerged strains. LAIV vaccines have shown the ability to protect against divergent strains in humans [32]. Challenge studies in several animal hosts also show the protective efficacy of LAIV vaccines against homologous and heterologous IAV infections [25,33]. Data from pig experiments indicate that intranasal LAIV vaccines induce mucosal immunity, e.g. virus-specific IgA and neutralizing antibodies in the upper and lower respiratory tract [22,23]. NI antibodies are potentially an important component of LAIV-induced immunity, which might

complement the neutralizing action of HA-binding antibodies, especially when HA antigenic drift occurs. Thus far, there are few published studies describing LAIV induction of NI antibodies in ferrets [34] and humans [8].

Homologous and cross-reactive NI antibodies in serum and respiratory specimens from pigs immunized with WT virus, LAIV, or WIV were measured. We determined that robust homologous serum NI responses were attained from each of the immunization regimens. Varying levels of NI activity against phylogenetically distinct N2 proteins within the 1998 lineage and more profoundly

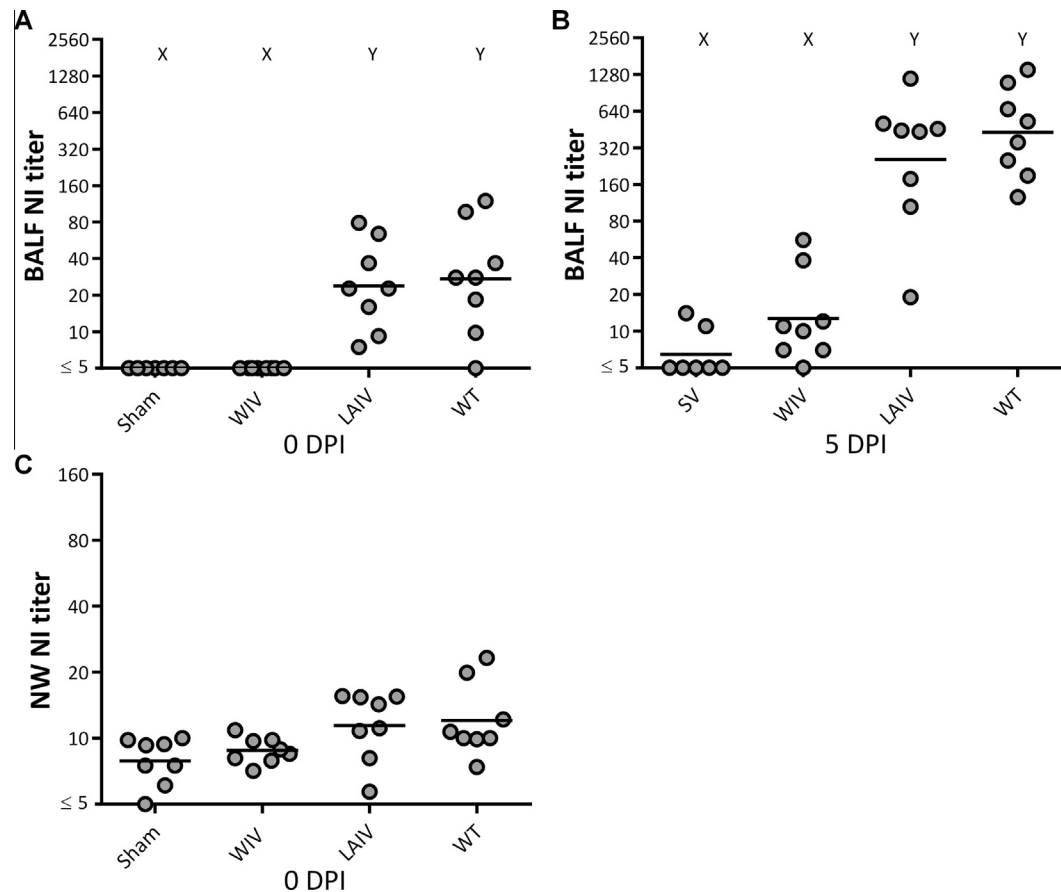


Fig. 4. Mucosal NI antibodies in pigs following vaccination or experimental infection. Three weeks after the secondary doses (six weeks post-primary vaccination), a subset of pigs were sacrificed. Bronchoalveolar lavage fluid (BALF) and nasal wash (NW) specimens were collected. Five days post-infection of the remaining pigs, they were euthanized and BALF specimens were collected. NI antibody titers to the homologous 1998-lineage N2_{MN08} protein were determined in pre-challenge BALF (A), post-challenge BALF (B), and pre-challenge NW (C). Data were analyzed statistically by ANOVA and Tukey's pairwise comparison test. Treatment groups marked with different letters had significantly different titers ($p < 0.05$).

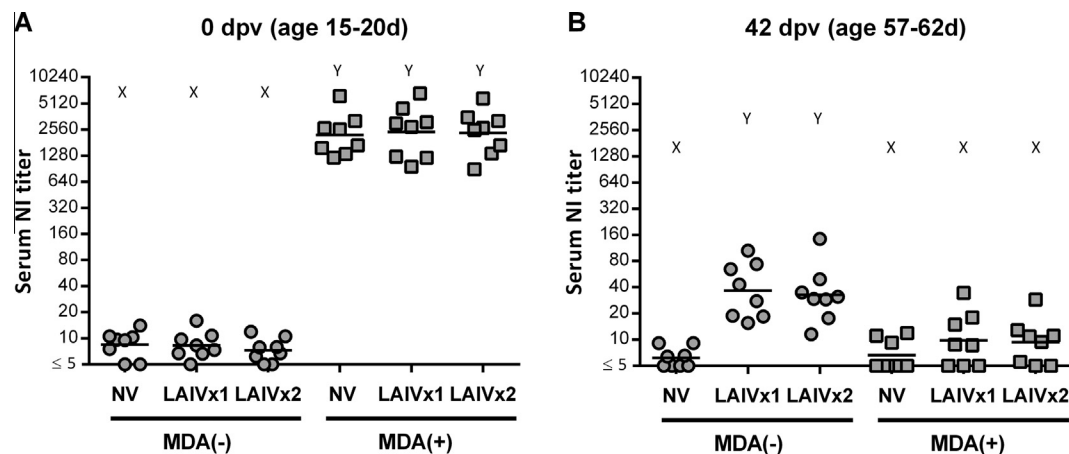


Fig. 5. Passive and adaptive serum NI antibodies measured in colostrum-fed piglets. Sows were vaccinated, before and during gestation, with a total of three doses of WIV; non-vaccinated control sows remained seronegative for IAV. Groups of piglets that suckled vaccinated sows are labeled as maternally-derived antibody positive [MDA(+)]. At 0 days post-vaccination (dpv) (15–20 days of age), piglets were bled for serum and designated groups were vaccinated intranasally with LAIV. Both of the LAIVx2 groups received secondary doses of LAIV at 21 dpv. Serum samples collected at 0 dpv (A) and 42 dpv (B) were tested for NI titers against N2_{TX98} protein, which is homologous to the sows' WIV vaccine and the piglets' LAIV vaccine. Data were analyzed statistically by ANOVA and Tukey's pairwise comparison test. Treatment groups marked with different letters had significantly different titers ($p < 0.05$).

between the 1998 and 2002 lineages were found in all immunized groups. The heterologous 1998-lineage N2, with 97% amino acid identity to the vaccine strain, demonstrated moderately lower cross-reactive NI titers. When the sera were assayed against a

2002-lineage N2, with about 92% amino acid identity, virtually no NI cross-reactivity could be detected. WIV vaccines containing N2 only of the 1998 lineage appear unlikely to elicit significant NI cross-reactivity against 2002-lineage strains.

Mucosal NI antibody responses were limited to the treatment groups exposed intranasally to live virus (LAIV or WT), despite the high serum NI titers in WIV vaccinated pigs. The WIV vaccine failed to induce respiratory tract NI antibodies; even 5 days after heterologous challenge infection the influx of NI antibodies to the lungs of WIV vaccinated pigs was modest. The LAIV and WT groups, in contrast, had robust NI activity in BAL fluid specimens and faint NI activity in NW, which is consistent with our previous data for whole virus-binding IgA [26,28]. Five days after heterologous challenge infection the LAIV and WT groups had pronounced increases in BAL fluid NI activity, presumably indicating an influx of antibodies from the circulation to the lung.

The mucosal response to a live virus includes functional NI antibodies, emphasizing that LAIV vaccines, as well as other platforms, should contain NA genes matched to the prominent circulating IAV strains in addition to updated HA genes. This is standard for human seasonal LAIV vaccines, as each component normally contains HA and NA of the approved reference strain. If circulating viruses then undergo HA antigenic drift while the NA antigen remains static [35], NA immunity may provide a level of protection. This is considerably different for swine vaccines in the US, where more viral lineages are co-circulating, strain updates to the commercial vaccines are infrequent, and no LAIV vaccines are presently licensed. Our results support inclusion of swine IAV strains with N2 genes of both the 1998 lineage and the 2002 lineage, since the two lineages co-circulate and have antigenic divergence. The presence of up-to-date NA antigens in LAIV or inactivated vaccines improves the likelihood of protection against antigenic drift variants and novel emerging strains.

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